PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Application of:
Mark G. Resnick

Serial No.: 10/806,494

Filed: March 3, 2004

For: METHODS FOR PREVENTING PHOTODAMAGED SKIN BY ADMINISTERING SELEGILINE OR DESMETHYLSELEGILINE

Group Art Unit: 1615

Examiner: L.S. Channavajjala

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APPEAL BRIEF

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In support of the appeal from the final rejection dated May, 27, 2005, Appellant now submits this Brief.

I. REAL PARTY IN INTEREST

The real party in interest of the patent application that is the subject of this appeal is the assignee, Somerset Pharmaceuticals, Inc.

II. RELATED APPEALS AND INTERFERENCES

Appeals are pending in the following two applications, which are also assigned to Somerset Pharmaceuticals, Inc.

1. Serial No. 10/885,221, Anthony R. DiSanto, filed July 6, 2004.
2. Serial No. 10/790,658, Cheryl D. Blume *et al.*, filed March 1, 2004.

The present application does not claim priority to the above applications or any other patents or applications in the same patent family as these two applications.

III. STATUS OF CLAIMS

Claims 31-53 are pending in the instant application, and Claims 31-53 stand rejected.

IV. STATUS OF AMENDMENTS

None

V. SUMMARY OF CLAIMED SUBJECT MATTER

Claims 31 and 43 are independent claims. Claim 31 is directed to a method of reducing photodamage to skin cells of a subject by administering a composition comprising selegiline and/or desmethylselegiline to the subject in an amount effective to reduce apoptosis of skin cells exposed to electromagnetic or ionizing radiation (*see, e.g.*, Specification, p.8, lines 19-20). Claim 43 is directed to a method of treating a subject for photodamaged skin, wherein the photodamage results from exposure to electromagnetic or ionizing radiation (*see, e.g.* Specification, p.3, lines 2-13), by administering a topical composition comprising selegiline and/or desmethylselegiline to the skin of the subject in an amount effective to reduce oxidative damage resulting from the exposure (*see, e.g.*, Specification, p.4, lines 8-13, 20, and 25-27).

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1. Whether claims 31-53 are unpatentable under 35 U.S.C. §103(a) as obvious over Tatton et al. (Neurology, 1996) in view of U.S. Patent No. 5,744,499 to Quash et al.

2. Whether claims 31-53 are unpatentable under 35 U.S.C. §103(a) as obvious over U.S. Patent No. 5,744,499 to Quash et al. in view of Tatton et al. (Neurology, 1996).

3. Whether claims 31-53 are unpatentable under 35 U.S.C. §103(a) as obvious over U.S. Patent No. 5,783,606 to Tatton in view of U.S. Patent No. 5,744,499 to Quash et al. and in view of Tatton et al. (Neurology, 1996).

VII. ARGUMENT

A. The rejection of claims 31-53 under 35 U.S.C. §103(a) as obvious over Tatton et al. (Neurology, 1996) in view of U.S. Patent No. 5,744,499 to Quash et al. is overcome

1. Claims 31-53

In the Final Office Action ("Final Action") issued May 27, 2005, claims 31-53 were rejected under 35 U.S.C. § 103(a) over Tatton et al. (Neurology, 1996) in view of U.S. Patent No. 5,744,499 to Quash et al. (Quash). The Examiner states that "Tatton et al teach deprenyl (same as selegiline) for reducing neuronal apoptosis caused by oxidative free radical damage and the reduction is mediated by a principal metabolite of deprenyl, desmethyldeprenyl (same as desmethylselegiline)" (Final Action, p.2), and that Quash "teaches modulation of apoptosis (induce or suppress) as a mechanism to prevent or provide treatment for photoinduced or chronological aging of skin and other related skin conditions." *Id.*

The Examiner cites this combination of references to assert that it would have been obvious "for one of ordinary skill in the art at the time of the instant invention to use the anti-apoptotic compounds (deprenyl and desmethyldeprenyl) of Tatton et al. (Neurology) for inhibiting or suppressing apoptosis in several dermal or epidermal conditions such as aging because Quash teaches that skin aging basically result from malfunctioning of skin mechanisms, especially due to apoptosis and suggests any species capable of modulating apoptosis can also prevent aging and its signs such as wrinkles." *Id.* at 3.

a. The Examiner has not established a *prima facie* case of obviousness

As Appellant demonstrates below, the teaching by Quash that any species capable of modulating apoptosis can also prevent aging and its signs is simply not true. Based on the skilled person's knowledge of the art (*i.e.*, knowledge of at least one anti-apoptotic compound that does not protect skin against photodamage), this person would have understood the fault of the premise stated in Quash, and therefore would not find the presently claimed subject matter obvious.

When setting forth an obviousness rejection, the MPEP clearly indicates that it is the Examiner who "bears the initial burden of factually supporting any *prima facie* conclusion of obviousness." MPEP § 2142. The MPEP sets forth that to establish a *prima facie* case of obviousness, three basic criteria must be met: (1) there must be a suggestion or motivation to combine the reference teachings either in the references themselves or in the general knowledge of one of ordinary skill in the art, (2) there must be a reasonable expectation of success, and (3) the references when combined must teach or suggest all the claim limitations. MPEP §§ 2142 & 2143. The Examiner bears the initial burden of factually supporting each of the three elements in order to make out a *prima facie* case of obviousness.

Although Appellant asserts that the obviousness rejection in the Final Action fails to satisfy any of the three criteria necessary to establish a *prima facie* case of obviousness, Appellant will only extensively address the Examiner's failure to show a reasonable expectation of success based on the combined references and her failure to show that one of ordinary skill in the art would have been motivated to combine the reference teachings. Since the Examiner has not met her burden of showing a *prima facie*

case of obviousness, Appellant is under no obligation to submit evidence of nonobviousness.

b. The obviousness rejection does not establish a reasonable expectation of success

The Examiner bears the initial burden of factually supporting a *prima facie* case of obviousness, which necessarily requires that the Examiner factually establish a reasonable expectation of success when combining the teachings of the references. MPEP § 2142. The Examiner however, cannot show the required "reasonable expectation of success," because one of skill in the art would not expect *any anti-apoptotic compound to treat photodamaged skin*, despite the suggestion made in Quash.

The Examiner relies on Quash to argue a reasonable expectation of success: "Quash teaches that skin aging basically result (sic) from malfunctioning of skin mechanisms, especially due to apoptosis and suggests any species capable of modulating apoptosis can also prevent aging and its signs such as wrinkles." Final Action, p.3. From this statement the Examiner concludes that "one of an ordinary skill in the art would have incorporated the compounds of Tatton et al. in the composition Quash and use for treating and/or combating photoinduced or chronological aging of the skin by modulating apoptosis because Tatton et al. suggests that the claimed compounds have the ability to reduce oxidative free radical initiated apoptosis." *Id.* at p.4.

But one of skill in the art would reject the suggestion made in Quash (and relied on by the Examiner for the obviousness rejection) because Quash does not provide sufficient factual support for the proposition that all anti-apoptotic agents would be expected to be effective in treating photodamage. For example, Quash is narrowly drawn

to modulating apoptosis with methional, malondialdehyde, or factors influencing the intracellular concentrations of methional or malondialdehyde. One of skill in the art would not consider the disclosure of only two anti-apoptotic agents to support the generalization made in Quash. Quash simply does not disclose a representative number of species to support this broad suggestion. Thus, the Examiner's reliance on Quash to factually establish a reasonable expectation of success by discussing the use of two compounds for modulating apoptosis out of the broad genus of anti-apoptotics is misplaced.

In addition to the minimal experimentation by Quash to support the broad suggestion relied upon by the Examiner, one of skill in the art would be aware that at least one well known anti-apoptotic compound, beta-carotene, has not been convincingly demonstrated in most clinical studies to protect against skin photodamage: "Undoubtedly, beta carotene is an important nutrient with powerful biological effects. Nevertheless, clinical studies as a whole have failed to persuade of an important role of beta-carotene as a photoprotector." *See* Biesalski et al., Arch. Biochem. Biophys. 389(1):1-6 (2001) ("Clinical trials demonstrated protective effects of beta-carotene against acute skin reactions [ ], but they failed to show any prevention of chronic photodamage [ ]. At present, both clinical and experimental data are highly inconsistent and some recent results also indicate the existence of potentially harmful effects of beta-carotene in UV-irradiated skin [ ]."). This knowledge would stop one of skill in the art from concluding that *any* anti-apoptotic compound can "prevent aging and its signs such as wrinkles." MPEP § 2142 requires that the Examiner show a *reasonable* expectation of success, not a mere possibility of success.

Beta-carotene has been shown in hepatic and brain cell lines to be an anti-apoptotic compound. Bagchi et al., Gen. Pharmac. 30(5):771-76 (1998); *see* also Ortmann *et al.*, Radiat. Res. 161(1):48-55 (2004) ("When given prior to irradiation, beta-carotene and vitamin E reduced the amount of radiation-induced apoptosis significantly..."). For example, Bagchi et al. found that beta-carotene was able to reduce TPA-induced hepatic and brain DNA fragmentation by 11%. *Id.* at p. 774. Since "[f]ragmentation of nuclear DNA is a biochemical hallmark of apoptosis," this finding demonstrates that beta-carotene has anti-apoptotic properties. *Id.*

Since the anti-apoptotic compound beta-carotene has not been shown to protect against skin photodamage, the suggestion by Quash relied on by the Examiner as the basis of the obviousness rejection is refuted. One of skill in the art would not have had a reasonable expectation of success in treating or promoting the healing of photodamaged skin with *any* anti-apoptotic compounds. Therefore, one of skill in the art would not have a reasonable expectation that selegiline or desmethylselegiline would have this claimed activity based solely on the knowledge that selegiline and desmethylselegiline have anti-apoptotic activity.

Given the faulty premise of Quash, the Examiner has not provided factual support establishing a reasonable expectation of success in utilizing desmethylselegiline and/or selegiline to treat photodamaged skin. Therefore, The Examiner has not met her burden to establish a *prima facie* case of obviousness.

c. The combination of Tatton et al. and Quash et al. does not suggest the desirability of Appellant's invention as a whole

If cited references can be properly combined, *O'Farrell* provides a three prong test for determining whether the combined references establish a *prima facie* case of obviousness. "The mere fact that references can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination." MPEP § 2143.01(III). *O'Farrell* held that for a combination of references to render a claimed invention obvious the references must provide to one of skill in the art:

(1) a detailed enabling methodology for practicing the claimed invention;
(2) a suggestion for modifying the prior art to practice the claimed invention; and
(3) evidence suggesting that the invention would be successful.

In re O'Farrell, 7 U.S.P.Q.2d 1673 (Fed. Cir. 1988).

There is no teaching or suggestion in Tatton et al. or Quash et al., either alone or in combination, of the desirability of the subject matter of the present disclosure as a whole. Therefore, these references cannot be properly combined. As admitted in the Final Action, Tatton et al. does not teach treating a subject for photodamaged skin; nor does this references suggest or motivate one of skill in the art that selegiline or desmethylselegiline can be used to treat photodamaged skin. In addition, Quash et al. does not teach selegiline or desmethylselegiline, nor does it support its suggestion that any species capable of modulating apoptosis can also treat photodamage. Thus, a skilled person would not assume that all anti-apoptotic compounds can treat photodamaged skin.

There is no teaching in the references cited by the Examiner or any other references of record that suggest or teach that selegiline and/or desmethylselegiline

would be useful for the treatment of photodamaged skin as disclosed in the present specification. Accordingly, these references do not suggest the desirability of Appellant's claimed subject matter as a whole and cannot be combined to render the pending claims obvious.

B. The rejection of claims 31-53 under 35 U.S.C. §103(a) as obvious over U.S. Patent No. 5,744,499 to Quash et al. in view of Tatton et al. (Neurology, 1996) is overcome

1. Claims 31-53

The Examiner's rejection of claims 31-53 under 35 U.S.C. § 103 over Quash et al. in view of Tatton et al. is based on the same two references discussed in Part A where Tatton et al. was the primary reference and Quash et al. was the secondary reference. Irrespective of the order in which these two references are presented, the disclosures remain the same.

For the same reasons set forth by Appellant in Part A, which show that the Examiner failed to establish both a motivation to combine and a reasonable expectation of success, the Examiner has failed to meet her burden for establishing a *prima facie* case of obviousness for the same references in reverse order. If the Examiner's reversed combination of these two references introduces any additional arguments that Appellant has not identified because they were not specifically set out by the Examiner, Appellant reserves the right to address those arguments in future correspondence related to the present application.

C. The rejection of claims 31-53 under 35 U.S.C. §103(a) as obvious over U.S. Patent No. 5,783,606 to Tatton (Tatton '606) in view of U.S. Patent No. 5,744,499 to Quash et al. in view of Tatton et al. (Neurology, 1996) is overcome

1. Claims 31-53

In the Final Action, the Examiner cited Tatton '606 to teach the use of "deprenyl and desmethyldeprenyl compounds for the treatment of glaucoma" and "administering deprenyl compositions in the forms of sprays, liquids, gels, pastes etc., for oral, nasal, topical or other routes." Final Action, p.3. The Examiner also stated that "it would have been obvious for one of ordinary skill in the art (sic) the time of the instant invention to use selegiline or desmethylselegiline of Tatton '606 for providing or combating aging in skin by inhibiting apoptosis because Quash teaches inhibiting apoptosis provides a treatment to aging skin and Tatton et al. teaches that deprenyl and desmethyldeprenyl are effective anti-apoptotic agents which reduce apoptosis caused by oxidative free radicals." *Id.* at p.4. The Examiner did not provide any additional discussion of Tatton '606.

The Examiner's addition of the Tatton '606 reference does not establish a reasonable expectation of success that any anti-apoptotic compound would be successful in treating photodamaged skin by combining Quash, Tatton et al. and Tatton '606. Since the Examiner failed to show a reasonable expectation of success for the combination of Quash and Tatton et al. as discussed in Part A, and since Tatton '606 does not provide any additional support for a reasonable expectation of success, the Examiner has failed to establish a *prima facie* case of obviousness based on the combination of Quash, Tatton et al. and Tatton '606.

C. CONCLUSION

Appellant respectfully submits that based on the foregoing observations and arguments, all pending claims listed in the Claims Appendix are non-obvious. It is therefore respectfully requested that the Board overturn each of the Examiner's rejections and allow the claims.

Respectfully submitted,

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VIII. CLAIMS APPENDIX

Claims 1-30 (Canceled).

Claim 31. (Previously presented) A method of reducing photodamage to skin cells of a subject comprising administering a composition comprising selegiline and/or desmethylselegiline to the subject in an amount effective to reduce apoptosis of skin cells exposed to electromagnetic or ionizing radiation.

Claim 32. (Previously presented) The method of claim 31, wherein the composition is a topical composition.

Claim 33. (Previously presented) The method of claim 32, wherein the topical composition is in a form selected from the group consisting of a cream, gel, transdermal patch, salve, lotion, and spray.

Claim 34. (Previously presented) The method of claim 32, wherein the topical composition comprises selegiline at a concentration of between 1×10^{-11} moles/liter and 1×10^{-3} moles/liter.

Claim 35. (Previously presented) The method of claim 32, wherein the topical composition comprises desmethylselegiline at a concentration of between 1×10^{-11} moles/liter and 1×10^{-3} moles/liter.

Claim 36. (Previously presented) The method of claim 31, wherein the composition comprises desmethylselegiline in the form of its R(-) enantiomer and the S(+) enantiomer is substantially absent.

Claim 37. (Previously presented) The method of claim 31, wherein the composition comprises desmethylselegiline in the form of its S(+)enantiomer and the R(-) enantiomer is substantially absent.

Claim 38. (Previously presented) The method of claim 31, wherein the composition further comprises a diluent or carrier comprising one or more compounds selected from the group consisting of water, suspending agents, thickeners, humectants, preservatives, emollients, emulsifiers, and film formers.

Claim 39. (Previously presented) The method of claim 31, wherein the selegiline is administered as the free base.

Claim 40. (Previously presented) The method of claim 31, wherein the selegiline is administered as a pharmaceutically acceptable acid addition salt.

Claim 41. (Previously presented) The method of claim 31, wherein the desmethylselegiline is administered as the free base.

Claim 42. (Previously presented) The method of claim 31, wherein the desmethylselegiline is administered as a pharmaceutically acceptable acid addition salt.

Claim 43. (Previously presented) A method of treating a subject for photodamaged skin, wherein the photodamage results from exposure to electromagnetic or ionizing radiation, comprising administering a topical composition comprising selegiline and/or desmethylselegiline to the skin of the subject in an amount effective to reduce oxidative damage resulting from exposure to electromagnetic or ionizing radiation.

Claim 44. (Previously presented) The method of claim 43, wherein the topical composition is in a form selected from the group consisting of a cream, gel, transdermal patch, salve, lotion, and spray.

Claim 45. (Previously presented) The method of claim 43, wherein the topical composition comprises selegiline at a concentration of between 1×10^{-11} moles/liter and 1×10^{-3} moles/liter.

Claim 46. (Previously presented) The method of claim 43, wherein the topical composition comprises desmethylselegiline at a concentration of between 1×10^{-11} moles/liter and 1×10^{-3} moles/liter.

Claim 47. (Previously presented) The method of claim 43, wherein the topical composition comprises desmethylselegiline in the form of its R(-) enantiomer and the S(+) enantiomer is substantially absent.

Claim 48. (Previously presented) The method of claim 43, wherein the topical composition comprises desmethylselegiline in the form of its S(+)enantiomer and the R(-) enantiomer is substantially absent.

Claim 49. (Previously presented) The method of claim 45, wherein the topical composition further comprises a diluent or carrier comprising one or more compounds selected from the group consisting of water, suspending agents, thickeners, humectants, preservatives, emollients, emulsifiers, and film formers.

Claim 50. (Previously presented) The method of claim 45, wherein the selegiline is administered as the free base.

Claim 51. (Previously presented) The method of claim 45, wherein the selegiline is administered as a pharmaceutically acceptable acid addition salt.

Claim 52. (Previously presented) The method of claim 45, wherein the desmethylselegiline is administered as the free base.

Claim 53. (Previously presented) The method of claim 45, wherein the desmethylselegiline is administered as a pharmaceutically acceptable acid addition salt.

IX. EVIDENCE APPENDIX

The following references were cited to the Examiner as support for the arguments in Appellant's response to the first Office Action dated September 29, 2004. Examiner acknowledged that Appellant's arguments had been fully considered in the Final Office Action dated May 27, 2005. (p.4, "Applicant's arguments filed 2-4-05 have been fully considered").

Exhibit 1. D. Bagchi et al., *Protective effects of grape seed proanthocyanidins and selected antioxidants against TPA-induced hepatic and brain lipid peroxidation and DNA fragmentation, and peritoneal macrophage activation in mice*, GENERAL PHARMACOLOGY, 30(5), p.771-76 (1998).

Exhibit 2. Hans K. Biesalski & U.C. Obermueller-Jevic, *UV light, beta-carotene and human skin--beneficial and potentially harmful effects*, ARCHIVES OF BIOCHEMISTRY & BIOPHYSICS, 389(1), p.1-6 (2001).

Exhibit 3. Elisabeth. K. Ortmann et al., *Effect of antioxidant vitamins on radiation-induced apoptosis in cells of a human lymphoblastic cell line*, Radiation Research, 161(1), p.48-55 (2004).

For the Board's convenience Appellant has included copies of the cited references.

X. RELATED PROCEEDINGS APPENDIX

None

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MINIREVIEW

UV Light, Beta-carotene and Human Skin—Beneficial and Potentially Harmful Effects

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Solar radiation is one of the most important environmental stress agents for human skin, causing sunburn, premature skin aging, and skin cancer. Beta-carotene is discussed to protect against photooxidative stress and thus prevent skin damage. Though beta-carotene has been successfully used against photosensitivity in patients with erythropoietic protoporphyria, its beneficial potential in normal skin is still uncertain. A number of experimental studies indicate protective effects of beta-carotene against acute and chronic manifestations of skin photodamage. However, most clinical studies have failed to convincingly demonstrate its beneficial effects so far. Nevertheless, intake of oral beta-carotene supplements before sun exposure has been recommended on a population-wide basis. Recent studies on skin cells in culture have revealed that beta-carotene acts not only as an antioxidant but also has unexpected prooxidant properties. At present, there is an ongoing debate on the protective or potentially harmful role of beta-carotene in human skin. © 2001 Academic Press

Key Words: **UV; carotenoids; beta-carotene; skin; oxidative stress; antioxidant; prooxidant.**

Beta-carotene has repeatedly been called a "sun protectant" and been credited with preventing solar damage to skin. Consequently, intake of oral supplements in times of increased sun exposure has been suggested to be beneficial (1–3) and is now very popular among sun seekers. The essential function of carotenoids protecting cells against photosensitized reactions was first hypothesized in the 1950s (4). Later, beta-carotene was found to prevent endogenous (chlorophyll) and exogenous photosensitization in bacteria, algae, and higher plants (5). Moreover, beta-carotene protected mice treated with hematoporphyrin (6) and humans suffering from photosensitivity to visible light (7). High-dose oral administration of beta-carotene has become a useful tool for therapy in patients with erythropoietic protoporphyria (EPP)[2] (8). This has lead to the suggestion that beta-carotene might also have protective properties in normal skin and thus prevent solar damage.

Overexposure to sunlight provokes acute sunburn reaction which clinically manifests itself as erythema. Chronic exposure to sun leads to premature skin aging ("photoaging") and increases the risk of both cutaneous melanoma and nonmelanoma skin cancer (NMSC) (9). Solar radiation has a strong oxidative component, and photooxidative stress has been directly linked to the onset of skin photodamage, as extensively reviewed by Fuchs (10). UVB radiation (280–320 nm) mainly damages DNA directly, due to an overlap with the absorption spectrum (11), and thus comprises a strong mutagenic potential. At the same time, it also has an oxidative component (12, 13). UVA radiation (320–400 nm), which contributes to up to 95% of total UV exposure (14), is not absorbed by DNA but it is a strong oxidant and considered the most important source of oxidative stress in human skin (15, 16).

The proposed beneficial effects of beta-carotene in skin have been mainly attributed to its antioxidant properties (2, 17–21). Experimental studies repeatedly found protection against UV-induced photodamage

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[2] Abbreviations used: EPP, erythropoietic protoporphyria; NMSC, nonmelanoma skin cancer; MED, minimal erythemal dose; HO1, heme oxygenase-1; DMBA, *N*-Benzyldime thylamin; TPA, Phorbol-12-myristat-13-acetate.

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such as erythema, immunosuppression, or skin cancer (22–26), though some authors found no beneficial effects (23, 27–30). So far, no clinical studies have been able to confirm these promising experimental results clearly (10). Clinical trials demonstrated protective effects of beta-carotene against acute skin reactions (2, 17, 31–33), but they failed to show any prevention of chronic photodamage (34–36). At present, both clinical and experimental data are highly inconsistent and some recent results also indicate the existence of potentially harmful effects of beta-carotene in UV-irradiated skin (37, 38). This review will briefly discuss the role of beta-carotene in human skin and its possible potential in protecting against the deleterious effects of solar radiation.

BETA-CAROTENE IN HUMAN SKIN

Carotenoids are part of the coloring system in human skin (39). Basal levels of beta-carotene in skin are usually rather low and were detected at ~0.03 to 0.4 nmol/g in wet tissue (1, 40) or at ~1.5 nmol/g when subcutaneous fat was included in samples (41). After consumption of a diet rich in carotenoids or oral supplementation, skin levels of beta-carotene are likely to increase, up to 17-fold over basal levels (1), which clinically manifests as yellowish complexion ("carotenodermia") (17, 31, 42–44).

In skin beta-carotene is mainly located in the epidermis (45, 46) where UVB radiation largely is absorbed (47). Beta-carotene does not absorb in the UVB range of light, and the oxidative component of UVB light is only weak. In dermal areas, where less beta-carotene is located, its antioxidant activity might be more important as they are the major target of UVA-induced oxidative stress. Near-ultraviolet light even reaches the cutaneous capillary system, indicating a possible field of action for beta-carotene in this compartment.

Exposure to sunlight reduces levels of beta-carotene in skin (1, 48). However, a single UV treatment did not significantly change concentrations of beta-carotene in skin but lycopene levels were decreased (49, 50). Overall, reduction of levels of beta-carotene and other carotenoids in skin might lower protection against UV damage. Consequently it may be hypothesized that increasing of beta-carotene concentrations before exposure to sunlight might provide a surplus and thus should reduce the risk of photodamage. Hata *et al.* (45) reported a correlation between carotenoid levels in skin and skin cancer. They found significantly lower carotenoid concentrations within perilesional, actinic keratosis and basal cell carcinoma sites when compared to skin from healthy subjects, and they suggested that reduced carotenoid levels in skin might predispose to the development of skin cancer.

Moderate carotenodermia is widely considered as beautiful and healthy complexion, particularly in infants, and the use of high doses of beta-carotene is considered relatively nontoxic (51). Nevertheless, it should be considered that a "safe range" of intracellular beta-carotene levels has not been determined yet, and it is not known whether beta-carotene accumulation in the skin might exert harmful side effects.

BETA-CAROTENE IN THE PREVENTION OF SKIN PHOTODAMAGE

The experience that beta-carotene inhibits photosensitized reactions in human skin (EPP) seemed to justify studies on the photoprotective effect of beta-carotene in normal skin. Initially, the results from clinical studies were rather disappointing. In 1972, Mathews-Roth and coworkers (33) published results of a clinical trial showing that long-term oral supplementation with beta-carotene (180 mg/day) increases the MED (minimal erythemal dose) to a small but not significant extent. In other studies beta-carotene did not significantly protect against UVA, UVB, and PUVA erythema after single (120 mg/day) or chronic ingestion of beta-carotene (90–180 mg/day), although values of carotenoids in the plasma reached levels that had been shown to be protective in patients with EPP and although levels in skin increased accordingly and even manifested carotenodermia (49, 52).

A few clinical trials found significant preventive effects of beta-carotene against acute photodamage. For example, we reported reduced erythema formation in subjects which had been supplemented with beta-carotene (30 mg/day) for 10 weeks before and also during exposure to sunlight (2). In this study we also observed that beta-carotene significantly increased the density of Langerhans cells prior to sun exposure and prevented their decrease after irradiation. Long-term supplementation with beta-carotene (30 mg/day) also protected from UVA-induced immunosuppression, as determined by delayed-type hypersensitivity tests which are accepted assays to evaluate the individual sensitivity to UV light (2, 32, 53). Stahl *et al.* (17) reported significantly reduced erythema formation following carotenoid supplementation with mainly beta-carotene (25 mg/day) over 12 weeks. Additional supplementation with 335 mg of vitamin E per day increased the protective effect of beta-carotene, but not to a significant extent. Recently, Lee *et al.* (31) published data from individuals who had been supplemented over 24 weeks with increasing doses of carotenoids (30–90 mg/day) consisting of mainly beta-carotene. In these subjects, the MED rose with carotenoid intake to a small but significant extent. In contrast to our results a dose of only 30 mg/day carotenoids, however, did not significantly alter the MED. Besides, serum lipid peroxida-

tion, determined with a lipid peroxidation activity assay, was significantly diminished during carotenoid supplementation.

The effect of beta-carotene on erythema prevention should be considered rather modest. Besides, it might be questioned whether reduction or even complete suppression of erythema formation as a physiological response to overexposure to sunlight is indeed beneficial or desirable.

Regarding chronic manifestations of skin photodamage, there is a lack of data on the prevention of photoaging by beta-carotene. A few data on skin cancer prevention have been published, nevertheless, the results from observational studies are inconsistent (54–62), and data from randomized, controlled trials are scarce (34, 63). The Physicians' Health Study and its follow-up analysis showed that beta-carotene supplementation (50 mg/day) over a period of 12 years has no effect on the development of a first NMSC (34) and other malignant neoplasms (36). This corresponds to results from the Nambour Skin Cancer Prevention Trial, which showed that beta-carotene supplementation (30 mg/day) over 4–5 years does not alter incidence of a first NMSC, neither with nor without sunscreen use (35). Results from a few observational studies showed an association between increased beta-carotene levels in plasma and a reduced risk of a first NMSC (54, 56), but most studies found no such effect (55, 57–59, 64). Obviously it is at present not possible to determine whether there is a relationship between plasma beta-carotene levels and risk of a first NMSC.

Concerning secondary prevention of skin cancer, Greenberg *et al.* (63) conducted the only randomized controlled clinical trial on the effect of beta-carotene supplementation on NMSC development. Patients who had recently had NMSC were given beta-carotene (50 mg/day) over a period of 5 years. Though plasma levels of beta-carotene rose 10-fold, no protection against the development of a new skin cancer was found.

It may be concluded that the clinical data available do not clearly show any preventive effectiveness of beta-carotene supplementation on skin cancer.

BETA-CAROTENE FUNCTION IN SKIN EXPOSED TO SUNLIGHT

At present, the basic molecular and pathophysiological aspects of the interaction of beta-carotene, skin, and UV light are poorly understood. The absorption spectra of carotenoids typically occur in the near ultraviolet and visible light region of 360–550 nm (65); however, Sayre and Black (66) reported that even in yellowish skin not enough beta-carotene was present to filter hazardous radiation to a significant extent.

Furthermore, prevention of direct DNA damage by beta-carotene has been regarded less likely (66). What seems more feasible is that beta-carotene acts as an antioxidant in the skin. Beta-carotene might provide protection against photosensitized reactions by quenching triplet sensitizers and singlet oxygen by energy transfer. Furthermore, beta-carotene might react with ROS such as oxygen radicals, peroxyl radicals, and singlet oxygen (67–70). A variety of experimental studies investigated the antioxidant function of beta-carotene in the skin *in vivo* and *in vitro.* In rodents, beta-carotene was found to reduce lipid peroxidation (20, 71, 72), and topical application of beta-carotene reduced *in vivo* chemiluminescence (73, 74). It has also been demonstrated that beta-carotene quenches singlet oxygen-mediated photochemical reactions in rodent skin (75–77).

In cultured skin cells, a few *in vitro* studies have investigated the antioxidant potential of beta-carotene. Beta-carotene decreased photoinactivation of the enzymes catalase and superoxide dismutase, as well as protein cross linking (78). Furthermore, beta-carotene protected against membrane damage and lipid peroxidation (21). In rat kidney fibroblasts beta-carotene diminished UVA-induced catalase deactivation and lipid peroxidation (19), and in embryonic lung fibroblasts beta-carotene protected from UVA-induced cell damage (18). In this study, positive synergy effects were observed when beta-carotene treatment was combined with vitamin E or vitamins E plus C. Interestingly, treatment with vitamin E or C alone had no protective effects, and in cells exposed to UVB light, the protective effect of beta-carotene was minor.

It should be taken into consideration that beta-carotene might also act through one of its metabolites. Beta-carotene is likely to be degraded by photochemically generated ROS ("photobleaching") (79). Thus, the decrease observed in beta-carotene levels in skin and plasma might be due to photodegradation or photoisomerization. Whether such metabolites occur in the skin *in vivo* following exposure to solar radiation must be elucidated. Furthermore, as a provitamin A carotenoid, beta-carotene might be metabolized to retinoids via central cleavage by the enzyme 15,15′-dioxygenase, which has recently been found in mouse skin (80). Retinoic acid is considered the biologically most effective metabolite and it has been used successfully for prevention and treatment of skin photodamage (81–83). In human skin, expression of 15,15′-dioxygenase or formation of retinoic acid from beta-carotene has not been demonstrated yet.

Beside a variety of experimental data which it is claimed to explain beneficial or even preventive aspects of beta-carotene in skin, a few experimental data exist which document more or less detrimental effects. In mice, aggravating effects on skin cancer were found (84–86). For example, beta-carotene increased the formation of skin papillomas in mice (85) treated with

DMBA and TPA. Even so, beta-carotene inhibited the conversion of papillomas to carcinomas which indicates a chemopreventive effect of beta-carotene (85, 87). Black recently stated (84) that future studies using carotenoid supplementation should be carried out with caution until interactions of carotenoids and repair mechanisms of radicals are clarified.

We investigated the effect of beta-carotene on the cellular stress response in dermal fibroblasts on the level of gene expression. Using the UVA induction of heme oxygenase-1 (HO-1) as an accepted marker for oxidative stress (88), we studied the effect of beta-carotene (0.5 and 5.0 μM) on the HO-1 expression in irradiated cells. HO-1 induction is attenuated by cellular antioxidants. Accordingly, singlet oxygen quencher beta-carotene should diminish HO-1 induction in irradiated cells which is a consequence of photochemical generation of singlet oxygen (89). Unexpectedly, beta-carotene strongly enhanced the UVA induction of HO-1, which indicates that beta-carotene can have a prooxidative effect. In our study, the prooxidative effect of beta-carotene observed could be entirely suppressed by vitamin E (25 μM), but only moderately by vitamin C (100 μM) (37). As cosupplementation of the cells with vitamin E abolished the UV-induced increase of HO-1 in beta-carotene-treated cells, we assume that beta-carotene acts as a prooxidant and consequently causes membrane lipid peroxidation which can be prevented by vitamin E. It might be questioned whether this enhanced HO-1 expression could have beneficial effects, as HO-1 has been called an "emergency inducible defense pathway" for protection against UVA radiation in dermal fibroblasts (90). HO-1 is part of an adaptive response to UVA radiation which leads to protection against oxidative membrane damage (91) and mediates immunoprotection (92). According to present knowledge, induction of HO-1 is due to oxidative stress, e.g., UVA radiation, hydrogen peroxide, hypoxia, and hyperoxia. Our results show that beta-carotene may act as an amplifier of UVA-induced oxidative stress and subsequent increase of HO-1 expression.

Jones *et al.* (38) also published data demonstrating a prooxidative effect of beta-carotene in dermal fibroblasts which supports our findings. Beta-carotene (10 μM) was found to increase UVA/B-generated oxidative stress, resulting in increased release of superoxide anions and lipid peroxidation. Furthermore, beta-carotene reduced cellular adaptation to UV irradiation with a rise in catalase and superoxide dismutase activities and increase in cellular glutathione content.

CONCLUSIONS

Undoubtedly, beta-carotene is an important micronutrient with powerful biological effects. Nevertheless, clinical studies as a whole have failed to persuade of an important role of beta-carotene as a photoprotector. The lack of an effect on skin cancer prevention in clinical trials might result from inadequate study protocols, as the duration of trials might have been too short and secondary prevention not the right target. However, even the results on acute manifestations of photodamage were ambiguous and mostly weak. In contrast to its effectiveness in the treatment of photosensitivity in patients with EPP, beta-carotene does not seem to be clinically beneficial as an oral sunscreen for healthy subjects. Beta-carotene might function as an antioxidant in human skin, but increasing levels in skin seems unlikely to modify the severity of skin photodamage. Experimental studies on the mechanism of action in skin cells *in vitro* have raised many questions and opened up a wide field of future research on the role of beta-carotene as a skin anti- or prooxidant.

It seems reasonable to assume that beta-carotene might combine both beneficial and detrimental effects in skin exposed to sunlight. The effects might depend on the biological endpoint investigated and the concentration of other antioxidants, e.g., vitamin E. In terms of using beta-carotene as a skin photoprotectant it should be pointed out that oral supplementation with beta-carotene as a single antioxidant might lead to an imbalance in the cutaneous antioxidant network and thus, as documented, exert possibly harmful effects on skin exposed to sunlight.

It seems questionable whether the present knowledge on beta-carotene action is sufficient to recommend intake of oral beta-carotene supplements or fortified food. Actually, it has been stated that there is at present no scientific evidence that high nutritional intake of beta-carotene from fruits and vegetables or a low-dose oral supplementation with beta-carotene might be harmful *in vivo* in general (93). From the data presented, we conclude that the use of single beta-carotene supplementation as an oral sun protectant should not be recommended at this time.

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Protective Effects of Grape Seed Proanthocyanidins and Selected Antioxidants against TPA-Induced Hepatic and Brain Lipid Peroxidation and DNA Fragmentation, and Peritoneal Macrophage Activation in Mice

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ABSTRACT. 1. The comparative protective abilities of a grape seed proanthocyanidin extract (GSPE) (25–100 mg/kg), vitamin C (100 mg/kg), vitamin E succinate (VES) (100 mg/kg) and β-carotene (50 mg/kg) on 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced lipid peroxidation and DNA fragmentation in the hepatic and brain tissues, as well as production of reactive oxygen species by peritoneal macrophages, were assessed.

2. Treatment of mice with GSPE (100 mg/kg), vitamin C, VES and β-carotene decreased TPA-induced production of reactive oxygen species, as evidenced by decreases in the chemiluminescence response in peritoneal macrophages by approximately 70%, 18%, 47% and 16%, respectively, and cytochrome *c* reduction by approximately 65%, 15%, 37% and 19%, respectively, compared with controls.

3. GSPE, vitamin C, VES and β-carotene decreased TPA-induced DNA fragmentation by approximately 47%, 10%, 30% and 11%, respectively, in the hepatic tissues, and 50%, 14%, 31% and 11%, respectively, in the brain tissues, at the doses that were used. Similar results were observed with respect to lipid peroxidation in hepatic mitochondria and microsomes and in brain homogenates.

4. GSPE exhibited a dose-dependent inhibition of TPA-induced lipid peroxidation and DNA fragmentation in liver and brain, as well as a dose-dependent inhibition of TPA-induced reactive oxygen species production in peritoneal macrophages.

5. GSPE and other antioxidants provided significant protection against TPA-induced oxidative damage, with GSPE providing better protection than did other antioxidants at the doses that were employed. GEN PHARMAC 30;5:771–776, 1998. **© 1998 Elsevier Science Inc.**

KEY WORDS. Oxidative stress, lipid peroxidation, DNA fragmentation, grape seed proanthocyanidin extract, vitamin C, vitamin E succinate, β-carotene, zinc L-methionine, Swiss-Webster mice, 12-O-tetradecanoylphorbol-13-acetate (TPA)

INTRODUCTION

Free radicals have been implicated in more than 100 disease conditions in humans, including arthritis, hemorrhagic shock, atherosclerosis, aging, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, tumor promotion and carcinogenesis, and AIDS (Ames, 1992; Halliwell, 1996; Halliwell and Cross, 1991; Halliwell *et al.*, 1992; Kehrer, 1993; Pitot and Dragan, 1991). Free radicals and their metabolites are increasingly recognized for their contribution to tissue injury leading to both initiation and promotion of multistage carcinogenesis (Pitot and Dragan, 1991). Recent studies have demonstrated that environmental pollutants, radiation, pesticides, various medications, contaminated water and deep-fried and spicy foods, as well as physical stress, exhibit the ability to produce enormous amounts of free radicals, resulting in oxidative deterioration of lipids, proteins and DNA, activation or procarcinogens, inhibition of cellular and antioxidant defense systems, depletion of sulfhydryls, altered calcium homeostasis, changes in gene expression and induction of abnormal proteins (Ames, 1992; Halliwell, 1996; Kehrer, 1993; Stohs and Bagchi, 1993).

Antioxidants/free-radical scavengers function as inhibitors at both initiation and promotion/propagation/transformation stages of tumor promotion/carcinogenesis and protect cells against oxidative damage (Halliwell, 1996; Halliwell and Cross, 1991; Halliwell *et al.*, 1992). The consumption of edible plants, fruits and vegetables has been demonstrated to prevent the occurrence of a number of diseases in humans and animals (Hocman, 1989). Vegetables, fruits and their seeds are rich sources of vitamins C and E, β-carotene and protease inhibitors, compounds that might protect the organism against free radical–induced injury and diseases (Hocman, 1989).

Proanthocyanidins, naturally occurring compounds widely available in fruits, vegetables, nuts, seeds, flowers and bark, are a group of polyphenolic bioflavonoids diverse in chemical structure, pharmacology and characteristics. Proanthocyanidins have been reported to exhibit a wide range of biological effects including antibacterial, antiviral, anti-inflammatory, antiallergic and vasodilatory actions (Afanas'ev *et al.*, 1989; Buening *et al.*, 1981; Kolodziej *et al.*, 1995). Furthermore, proanthocyanidins have been reported to inhibit lipid peroxidation, platelet aggregation and capillary perme-

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ability and fragility and to modulate the activity of enzyme systems including cyclooxygenase and lipooxygenase (Bors and Saran, 1987; Kolodziej *et al.*, 1995). Proanthocyanidins are believed to be nontoxic. If they are absorbed and biologically active *in vivo*, they may prevent free radical–mediated cytotoxicity and lipid peroxidation and protect low-density lipoproteins from oxidation (Frankel *et al.*, 1993; Kinsella *et al.*, 1993).

A variety of proanthocyanidins have been shown to prevent the growth of breast cancer cells and to inhibit the enzymes taking part in the replication of rhino viruses (common cold) and HIV viruses (Hocman, 1989). The potential of isoflavones and lignans, also known as phytoestrogens, for preventing the development of hormone-dependent cancers such as breast and prostate cancer is attributed to their being weak estrogens (Hocman, 1989). Proanthocyanidins may exert these effects as antioxidants, potent free-radical scavengers and chelators of toxic heavy metals (Chen *et al.*, 1996; Rice-Evans *et al.*, 1996).

In this study, we have assessed the comparative protective abilities of a grape seed proanthocyanidin extract (GSPE) with vitamin C, vitamin E succinate (VES) and β-carotene *in vivo* against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)–induced lipid peroxidation and DNA fragmentation in hepatic and brain tissues as well as against production of oxygen free radicals in peritoneal macrophages of mice.

MATERIALS AND METHODS

Chemicals

A commercially available dried, powdered GSPE (batch no. AV 609016) was obtained from InterHealth Nutritionals Inc. (Concord, CA). All other chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, MO) and were of analytical grade or the highest grade available.

Animals and treatment

Female Swiss-Webster mice (20–25 g) were obtained from Sasco (Omaha, NE). The animals were housed in a controlled environment at 25°C with a 12-hr light and 12-hr dark cycle and were acclimated for at least 3–5 days before use. All animals were allowed free access to food (Purina Rodent Lab Chow No. 5001) and tap water. VES and β-carotene were dissolved in corn oil, whereas GSPE and vitamin C were dissolved in water. GSPE (25–100 mg/kg), vitamin C (100 mg/kg), VES (100 mg/kg) and β-carotene (50 mg/kg) were orally administered to groups of animals with the use of a feeding needle for 7 consecutive days. All treatments were conducted daily in the morning between 7:30 A.M. and 8:30 A.M. All groups of mice received an intraperitoneal (IP) injection of 1 ml of 3% thioglycollate (DIFCO Laboratories, Detroit, MI) broth 3 days before TPA treatment to elicit peritoneal macrophages (Witz and Czerniecki, 1989). TPA was administered on the 8th day 2 hr after the antioxidant treatment. Groups of mice were individually treated IP with 0.1 μg TPA diluted in 1 ml of sterile phosphate-buffered saline (PBS) to induce an oxidative stress and were killed 2 hr posttreatment by cervical dislocation. Control animals received the PBS buffer. The peritoneal macrophage cells were isolated, the hepatic and brain tissues were quickly removed and the subcellular fractions were obtained as described elsewhere (Bagchi and Stohs, 1993). An approval (ARC no. 0313) from the Creighton University Animal Research Committee was obtained for this project.

Chemiluminescence assay

Chemiluminescence, as an index of reactive oxygen species production, was measured in a Chronolog Lumivette luminometer (Chronolog Corp., Philadelphia, PA). The assay was conducted in 3-ml glass minivials. The vials were incubated at 37°C before measurement, and the background chemiluminescence of each vial was checked before use. Samples were preincubated at 37°C for 15 min, and 4 μM luminol was added to enhance chemiluminescence. All additions to the vials as well as chemiluminescence-counting procedures were performed under dim lighting conditions. Results were presented as counts per unit time minus background. Chemiluminescence was monitored for 6 min at continuous 30-sec intervals (Bagchi and Stohs, 1993).

Cytochrome c reduction assay

Superoxide anion production by peritoneal macrophages was measured by the cytochrome c reduction assay of Babior *et al.* (1973). The reaction mixtures contained 1 ml of macrophages (3×10^6 cells/ml) and 0.05 mM cytochrome c. The reaction mixtures were incubated for 15 min at 37°C. The reactions were terminated by placing the reaction mixtures in ice. The mixtures were centrifuged at 1,500*g* for 10 min at 4°C, and the supernatant fractions were transferred to clean tubes for subsequent spectrophotometric measurement at 550 nm. Absorbance values were converted into nanomoles of cytochrome c reduced by using the extinction coefficient of 2.1×10^4/M/cm/15 min (Bagchi and Stohs, 1993).

Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) associated with hepatic mitochondria and microsomes, as well as brain homogenates from control and treated animals were determined as an index of lipid peroxidation according to the method of Buege and Aust (1984) and as previously published by us (Bagchi and Stohs, 1993). Malondialdehyde was used as the standard. Absorbance values were measured at 535 nm, and an extinction coefficient of 1.56×10^5/M/cm was used.

DNA fragmentation

Liver and brain samples were homogenized in lysis buffer (5 mM Tris-HCl, 20 mM EDTA, 0.5% Triton X-100, pH 8.0). Homogenates were centrifuged at 27,000*g* for 20 min to separate intact chromatin in the pellets from fragmented DNA in the supernatant fractions. Pellets were resuspended in 0.5 N perchloric acid, and 5.5 N perchloric acid was added to supernatant samples to reach a concentration of 0.5 N. Samples were heated at 90°C for 15 min and centrifuged at 1,500*g* for 10 min to remove protein. Resulting supernatants were reacted with diphenylamine for 16–20 hr at room temperature, and absorbance was measured at 600 nm. DNA fragmentation is expressed as a percentage of total DNA appearing in the supernatant fractions. Treatment effects are reported as a percentage of control fragmentation (Ray *et al.*, 1993).

Statistical analysis

Data for each group were subjected to analysis of variance. Scheffe's S method was used as the *post hoc* test. The data are expressed as the mean±standard deviation of four animals. The level of statistical significance employed in all cases was $P<0.05$.

RESULTS

Production of reactive oxygen species

TPA-induced *in vivo* production of oxygen free radicals in the peritoneal macrophages of mice was assessed by luminol-enhanced chemiluminescence and cytochrome c reduction assays. The results

TABLE 1. Production of reactive oxygen species by peritoneal macrophages based on chemiluminescence response and cytochrome c reduction after treatment of mice with TPA, and the comparative scavenging abilities of GSPE and selected antioxidants

Sample	Chemiluminescence (CPM/3 × 10^6 cells)	Cytochrome c reduction (nmoles/15 min/3 × 10^6 cells)
Control	995 ± 156a	4.55 ± 0.43a
Corn oil	937 ± 88a	4.35 ± 0.17a
Vitamin C (100 mg/kg)	1114 ± 141a	5.27 ± 0.50b
VES (100 mg/kg)	1771 ± 139b	9.10 ± 0.65c
Vitamin C + VES (100 mg/kg each)	1724 ± 140b	9.02 ± 0.58c
β-Carotene (50 mg/kg)	1198 ± 118a	3.93 ± 0.70a
GSPE (100 mg/kg)	1306 ± 94a	5.07 ± 0.58b
TPA	6031 ± 591c	26.61 ± 1.60d
TPA + vitamin C (100 mg/kg)	5081 ± 335d	22.67 ± 2.36d
TPA + VES (100 mg/kg)	3455 ± 321e	18.03 ± 0.83e
TPA + vitamin C + VES (100 mg/kg each)	2934 ± 132e	14.24 ± 1.52f
TPA + β-carotene (50 mg/kg)	5015 ± 199d	21.81 ± 1.38d
TPA + GSPE (25 mg/kg)	3592 ± 211e	18.21 ± 1.86e
TPA + GSPE (50 mg/kg)	2732 ± 99e	12.65 ± 1.41f
TPA + GSPE (100 mg/kg)	1724 ± 268b	8.26 ± 0.84c

Female Swiss-Webster mice were treated with a single dose of 0.1 μg TPA after treatment with antioxidant(s) for 7 days. Peritoneal exudate cells (primarily macrophages) were analyzed for enhanced chemiluminescence and cytochrome c reduction. Each value represents the mean ± SD of four mice. Values with nonidentical lowercase letters are significantly different ($P < 0.05$).

of the chemiluminescence and cytochrome c reduction assays for the production of reactive oxygen species by peritoneal exudate cells (primarily macrophages) are presented in Table 1.

The chemiluminescence produced by peritoneal macrophages from TPA-treated animals rapidly rises, reaching a maximum between 3 and 4 min, whereas macrophages from control animals reach a peak chemiluminescence at 3 min (data not shown). No significant increases in chemiluminescence were observed after treatment of the mice with either GSPE, vitamin C or β-carotene (Table 1). An approximately 1.8-fold increase in chemiluminescence was observed in the peritoneal macrophages of animals treated with VES alone. The succinate moiety has been previously shown to be responsible for this effect (Bagchi *et al.*, 1993).

A 6.1-fold increase in chemiluminescence was observed after treatment of the animals with TPA. Administration of 25, 50 and 100 mg GSPE/kg to the animals for 7 consecutive days decreased the TPA-induced chemiluminescence in the peritoneal macrophages by 40%, 55% and 71%, respectively, compared with control values. Thus, a dose-dependent inhibition was demonstrated by GSPE. Pretreatment of animals with vitamin C (100 mg/kg), VES (100 mg/kg), a combination of vitamin C plus VES (100 mg/kg each) and GSPE (100 mg/kg) decreased the TPA-induced chemiluminescence by 16%, 43%, 51% and 71%, respectively, compared with control samples. Administration of β-carotene (50 mg/kg) and GSPE (50 mg/kg) for 7 consecutive days decreased the TPA-induced chemiluminescence response by 17% and 55%, respectively, relative to the control values.

The effect of TPA on the production of superoxide anion by peritoneal macrophages (determined by the cytochrome c reduction assay) also is presented in Table 1. The data are expressed as nanomoles of cytochrome c reduced 3×10^6 cells/15 min. GSPE, vitamin C and β-carotene had no effect on superoxide anion production in the absence of TPA. As previously noted with chemiluminescence, VES produced a significant increase (approximately 2.0-fold) in superoxide anion production (Table 1).

TPA administration increased the production of superoxide anion on the basis of cytochrome c reduction compared with the cells from untreated animals by 5.9-fold (Table 1). GSPE induced a dose-dependent inhibition of the TPA-induced cytochrome c reduction. Administration of 25, 50 and 100 mg GSPE/kg to the animals for 7 consecutive days decreased TPA-induced cytochrome c reduction by 32%, 53% and 69%, respectively, which still represented approximately 4.0-, 2.8- and 1.8-fold increases, respectively, above the control values. Pretreatment of animals with vitamin C (100 mg/kg), VES (100 mg/kg), a combination of vitamin C plus VES (100 mg/kg each) and GSPE (100 mg/kg) decreased TPA-induced cytochrome c reduction by approximately 15%, 32%, 47% and 69%, respectively, compared with control samples. Administration of β-carotene (50 mg/kg) and GSPE (50 mg/kg) for 7 consecutive days decreased TPA-induced cytochrome c reduction by approximately 18% and 48%, respectively, relative to the control values.

Lipid peroxidation

The effects of TPA and antioxidants on lipid peroxidation in hepatic mitochondria and microsomes and in brain homogenates are summarized in Table 2. No significant increases in lipid peroxidation were observed with GSPE, vitamin C or β-carotene. Approximately 1.4-, 1.2- and 1.2-fold increases in lipid peroxidation were observed in the hepatic mitochondria, hepatic microsomes, and brain homogenates, respectively, compared with control animals after treatment of the animals with VES, similar to previously reported observations (Bagchi *et al.*, 1993).

After treatment of mice with TPA, increases in lipid peroxidation of 2.7-, 2.9- and 3.1-fold were observed in hepatic mitochondria, hepatic microsomes and brain homogenates, respectively, compared with control values. Administration of 25, 50 and 100 mg GSPE/kg for 7 days to these animals decreased TPA-induced hepatic mitochondrial lipid peroxidation by 37%, 41% and 46%, respectively; in the hepatic microsomal fractions, decreases of 47%, 55% and 59% were observed, respectively, compared with control values. Approximately 46%, 53% and 61% decreases were demonstrated by GSPE

TABLE 2. TPA-induced lipid peroxidation in hepatic mitochondria and microsomes, and in brain homogenates of mice, and the comparative protective abilities of GSPE and selected antioxidants

	Lipid peroxidation (nmoles MDA/mg of protein)		
	Liver		Brain
	Mitochondria	Microsomes	Whole homogenate
Control	2.17 ± 0.24a	2.76 ± 0.22a	1.62 ± 0.13a
Corn oil	2.30 ± 0.11a	2.59 ± 0.35a	1.69 ± 0.13a
Vitamin C (100 mg/kg)	2.38 ± 0.19a	2.85 ± 0.25a	1.68 ± 0.10a
Vitamin E succinate (VES) (100 mg/kg)	3.05 ± 0.14b	3.19 ± 0.29b	1.93 ± 0.17b
Vitamin C + (VES) (100 mg/kg each)	3.01 ± 0.17b	3.11 ± 0.15b	1.95 ± 0.10b
β-Carotene (50 mg/kg)	2.11 ± 0.12a	2.83 ± 0.11a	1.55 ± 0.12a
GSPE (100 mg/kg)	2.32 ± 0.14a	2.67 ± 0.32a	1.71 ± 0.22a
TPA	5.81 ± 0.34c	8.12 ± 0.84c	4.95 ± 0.32c
TPA + Vitamin C (100 mg/kg)	5.12 ± 0.34c	7.02 ± 0.42c	4.32 ± 0.23c
TPA + VES (100 mg/kg)	3.71 ± 0.39d	4.29 ± 0.44d	2.71 ± 0.49d
TPA + Vitamin C + VES (100 mg/kg each)	3.54 ± 0.49b,d	3.81 ± 0.42d,e	2.57 ± 0.35d
TPA + β-Carotene	5.41 ± 0.38c	7.17 ± 0.46c	4.54 ± 0.22c
TPA + GSPE (25 mg/kg)	3.68 ± 0.39d	4.33 ± 0.49d	2.69 ± 0.28d
TPA + GSPE (50 mg/kg)	3.43 ± 0.22d	3.65 ± 0.25e	2.33 ± 0.24d
TPA + GSPE (100 mg/kg)	3.13 ± 0.27b	3.31 ± 0.28b	1.94 ± 0.40b

Swiss-Webster mice were treated with a single dose TPA after receiving antioxidant(s) for 7 days. Thiobarbituric acid reactive substances (TBARS) as an index of lipid peroxidation were determined on hepatic mitochondria and microsomes and on brain homogenates from control and treated animals. Malondialdehyde was used as the standard. Each value represents the mean ± SD of four mice. Values with nonidentical lowercase letters are significantly different ($P < 0.05$).

against TPA-induced lipid peroxidation in the brain homogenates at these same concentrations.

Administration of vitamin C (100 mg/kg), VES (100 mg/kg), a combination of vitamin C and VES (100 mg/kg each) and GSPE (100 mg/kg) for 7 days decreased TPA-induced hepatic mitochondrial lipid peroxidation by 12%, 36%, 39% and 46%, respectively, compared with control values, and 14%, 47%, 53% and 59% decreases, respectively, were observed in the hepatic microsomes. After treatment of the animals with these same antioxidants, 13%, 45%, 48% and 61% decreases, respectively, were observed against TPA-induced lipid peroxidation in brain homogenates. Administration of β-carotene (50 mg/kg) decreased TPA-induced hepatic mitochondrial and microsomal lipid peroxidation by approximately 7% and 12%, respectively; under these same conditions, an 8% decrease was observed in brain homogenate lipid peroxidation, compared with control values.

DNA fragmentation

Programmed cell death (apoptosis) has been identified as a selective process of physiological cell deletion. Apoptosis is accompanied by condensation of cytoplasm, loss of plasma membrane microvilli, condensation and fragmentation of nuclei and extensive degradation of chromosomal DNA. Fragmentation of nuclear DNA is a biochemical hallmark of apoptosis (Schwartzman and Cidlowski, 1993).

TPA-induced DNA fragmentation in hepatic and brain tissues is summarized in Table 3, and the comparative protective abilities of various antioxidants are presented. TPA-induced 2.2- and 2.5-fold increases in DNA fragmentation in the hepatic and brain tissues of mice, respectively, compared with controls. No significant increases in DNA fragmentation were observed with GSPE, vitamin C or β-carotene. Approximately 1.3- and 1.4-fold increases in DNA fragmentation were observed in the liver and brain tissues, respectively, compared with control animals after treatment of the animals with VES alone, similar to previous observations (Bagchi *et al.*, 1993).

A dose-dependent protective ability against TPA-induced DNA fragmentation was demonstrated by GSPE. Administration of 25, 50 and 100 mg GSPE/kg to the animals for 7 days decreased TPA-induced hepatic DNA fragmentation by 36%, 42% and 47%, respectively, compared with control values, and DNA fragmentation decreased by approximately 32%, 44% and 50% in the brain tissues at these same concentrations. Pretreatment of animals with vitamin C (100 mg/kg), VES (100 mg/kg), a combination of vitamin C plus VES (100 mg/kg each) and GSPE (100 mg/kg) decreased TPA-induced hepatic DNA fragmentation by 10%, 30%, 38% and 47%, respectively; under these same conditions, DNA fragmentation was reduced by 14%, 31%, 40% and 50% in brain tissues, respectively, compared with control samples. Administration of β-carotene (50 mg/kg) for 7 days reduced TPA-induced hepatic and brain DNA fragmentation by 11%, relative to the respective control values.

DISCUSSION

Proanthocyanidins are a group of polyphenolic bioflavonoids ubiquitously found in fruits and vegetables. Proanthocyanidins have gained recent interest because of their broad pharmacological activity and therapeutic potential (Chen *et al.*, 1996; Hanefield and Herrmann, 1976; Masquelier *et al.*, 1979). Putative therapeutic effects of many traditional medicines may be ascribed to the presence of bioflavonoids (Brandi, 1992; Chen and Chan, 1996; Havsteen, 1983). The chemical properties of bioflavonoids in terms of the availability of the phenolic hydrogens as hydrogen-donating radical scavengers and singlet oxygen quenchers predict their antioxidant activity (Chen *et al.*, 1996; Rice-Evans *et al.*, 1996). For a proanthocyanidin or a bioflavonoid to be defined as an antioxidant, it must satisfy two basic conditions: (1) when present in low concentrations relative to the substrate to be oxidized, it can delay, retard, or pre-

TABLE 3. TPA-induced DNA fragmentation in the hepatic and brain tissues, and the comparative protective abilities of GSPE and selected antioxidants

Sample	Liver (%)	Brain (%)
Control	2.04 ± 0.30a	1.77 ± 0.28a
Corn oil	2.19 ± 0.31a	1.73 ± 0.22a
Vitamin C (100 mg/kg)	2.16 ± 0.34a	2.19 ± 0.31a,b
VES (100 mg/kg)	2.63 ± 0.24b	2.47 ± 0.36b
Vitamin C + VES (100 mg/kg each)	2.54 ± 0.36b	2.33 ± 0.31b
β-Carotene (50 mg/kg)	2.13 ± 0.22a	1.97 ± 0.26a
GSPE (100 mg/kg)	2.16 ± 0.31a	1.89 ± 0.31a
TPA	4.57 ± 0.51c	4.41 ± 0.28c
TPA + vitamin C (100 mg/kg)	4.12 ± 0.31c	3.80 ± 0.38d
TPA + VES (100 mg/kg)	3.18 ± 0.45d	3.03 ± 0.26e
TPA + vitamin C + VES (100 mg/kg each)	2.83 ± 0.23b,d	2.66 ± 0.21b
TPA + β-carotene (50 mg/kg)	4.06 ± 0.29c	3.92 ± 0.20d
TPA + GSPE (25 mg/kg)	2.94 ± 0.51b,d	3.00 ± 0.16c
TPA + GSPE (50 mg/kg)	2.67 ± 0.21b	2.49 ± 0.24b
TPA + GSPE (100 mg/kg)	2.43 ± 0.21b	2.22 ± 0.19b

Female Swiss-Webster mice were treated with a single dose of 0.1 μg TPA after receiving antioxidant(s) for 7 days. DNA fragmentation was measured spectrophotometrically by using Burton's reagent. Each value represents the mean ± SD of four mice. Values with nonidentical lowercase letters are significantly different ($P < 0.05$).

vent autooxidation or free radical–mediated oxidative injury; and (2) the resulting product formed after scavenging must be stable through intramolecular hydrogen bonding on further oxidation (Shahidi and Wanasundara, 1992).

The biological, pharmacological and medicinal properties of the bioflavonoids and proanthocyanidins have been extensively reviewed (Jovanovic *et al.*, 1994; Rice-Evans *et al.*, 1996; Suzuki, 1993). Flavonoids and other plant phenolics are reported to possess, in addition to their free-radical scavenging and antioxidant activity, multiple biological activities including vasodilatory, anticarcinogenic, anti-inflammatory, antibacterial, immune-stimulating, antiallergic, antiviral and estrogenic activities, as well as being inhibitors of the enzymes phospholipase A_2, cyclooxygenase and lipooxygenase (Rice-Evans *et al.*, 1996; Salah *et al.*, 1995).

The presence of various phenolic compounds, including phenoldienones, epicatechin, epigallocatechin, epigallocatechin gallate, ferulic acid, caffeic acid, *p*-coumaric acid, kaempferol, quercetin and myricetin, have been well established in proanthocyanidin extracts (Gonzalez *et al.*, 1982; Hanefield and Herrmann, 1976; Masquelier *et al.*, 1979; Rice-Evans *et al.*, 1996). It is well known that diets rich in fresh fruits and vegetables are protective against cardiovascular diseases and other oxidative stress–induced diseases and disorders including cancer (Chen and Chan, 1996; Halliwell, 1996; Halliwell *et al.*, 1992; Hocman, 1989). These chemoprotective properties have been attributed, in large part, to the presence of antioxidant nutrients vitamin C, vitamin E, β-carotene and mineral micronutrients. However, plant phenolics such as the bioflavonoids, proanthocyanidins and phenylpropanoids also may play a significant role. The proanthocyanidins or polyphenolic bioflavonoids may act as antioxidants or by other mechanisms, contributing to anticarcinogenic or chemoprotective actions or both.

In this study, the protective abilities of GSPE, a commercially available grape seed proanthocyanidin extract, vitamin C, VES, a combination of vitamin C plus VES and β-carotene were assessed on TPA-induced oxidative tissue and DNA damage in the hepatic and brain tissues, as well as activation of peritoneal macrophages. The production of reactive oxygen species by peritoneal macrophages was assessed by measuring chemiluminescence and cytochrome c reduction (Table 1). Cytochrome c reduction is a specific test for superoxide anion production (Ritchey *et al.*, 1981), whereas chemiluminescence is a general assay for the production of reactive oxygen species (Fisher and Adams, 1985). These assays clearly demonstrate the production of reactive oxygen species by peritoneal macrophages after administration of TPA and the comparative protective abilities of GSPE, vitamin C, a combination of vitamin C plus VES and β-carotene. GSPE demonstrated the best protection in the chemiluminescence assay compared with vitamin C, VES or β-carotene at the doses that were used. The combination of vitamin C and VES demonstrated better protection compared with the individual vitamins alone, which may be the result of regeneration of vitamin E from its oxidized form by vitamin C (Buettner, 1993). Similar results were obtained in the cytochrome c reduction assay (Table 1). These data indicate that GSPE as well as other antioxidants may be useful in preventing the *in vivo* production of reactive oxygen species.

Lipid peroxidation was assessed in the hepatic mitochondria and microsomes and in brain homogenate (Table 2); DNA fragmentation data for hepatic and brain tissues are presented in Table 3. Lipid peroxidation and DNA fragmentation serve as indicators of oxidative tissue damage. DNA fragmentation is believed to be a biochemical hallmark of apoptosis (programmed cell death), which plays a major role in developmental biology and in the maintenance of homeostasis in vertebrates (Schwartzman and Cidlowski, 1993). GSPE exhibited the best protection against TPA-induced hepatic mitochondrial and microsomal lipid peroxidation compared with the other antioxidants tested at the doses that were used (Table 2). A combination of vitamin C plus VES exerted better protection than did the corresponding individual vitamins. All antioxidants that were tested ameliorated TPA-induced increases in lipid peroxidation and DNA fragmentation in both brain and liver (Tables 2 and 3), with GSPE exhibiting the best protection compared with the other antioxidants.

These *in vivo* experiments demonstrate that GSPE is a better scavenger of free radicals and inhibitor of oxidative tissue damage than vitamin C, VES, a combination of vitamin C plus VES and β-carotene at the doses that were used. The results clearly demon-

strate that GSPE significantly attenuates TPA-induced oxidative stress in hepatic and brain issues, as well as in peritoneal exudate cells (primarily macrophages). These data confirm that GSPE can significantly attenuate TPA-induced lipid peroxidation and DNA fragmentation in the hepatic and brain tissues, as well as the enhanced production of oxygen free radicals in peritoneal macrophages. Furthermore, the results indicate that GSPE is bioavailable to vital target organs, including the liver and brain tissues and peritoneal exudate cells, and may therefore be useful in preventing the production of reactive oxygen species and oxidative tissue damage *in vivo*.

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Effect of Antioxidant Vitamins on Radiation-Induced Apoptosis in Cells of a Human Lymphoblastic Cell Line

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Modulating the amount of radiation-induced apoptosis by administering antioxidant vitamins offers a possible way to influence radiation-induced side effects in normal tissues. Therefore, we investigated the effect of beta-carotene, vitamin C and alpha-tocopherol on radiation-induced apoptosis in cells in culture. Human T-lymphoblastic MOLT-3 cells were irradiated with a dose of 3 Gy 1 h after or immediately prior to the addition of vitamins in three concentrations (0.01 μ*M*, 1 μ*M* and 100 μ*M*). Eight hours later, apoptosis was scored morphologically by staining the nuclear DNA with Hoechst 33342. When given prior to irradiation, beta-carotene and vitamin E reduced the amount of radiation-induced apoptosis significantly at concentrations of 0.01 μ*M* and 1 μ*M*. In contrast, vitamin C did not show any protective effect when given at these two concentrations and caused a slight but significant radiosensitization at 100 μ*M*. At 0.01 μ*M*, all combinations of two vitamins showed a protective effect. This was also observed for the combination of all three vitamins at concentrations of 0.01 and 1 μ*M*. When given immediately after irradiation, each of the three vitamins showed a protective effect at 0.01 μ*M*. In addition, the combination of alpha-tocopherol and vitamin C reduced radiation-induced apoptosis slightly when given at 1 μ*M*. In all other cases, no statistically significant modulation of radiation-induced apoptosis was observed. In our experimental system, the protective effect of beta-carotene and vitamin E was dependent on concentration and occurred only in the micromolar and sub-micromolar concentration range, while vitamin C alone, but not in combinations, had a sensitizing effect, thus arguing for a careful consideration of vitamin concentrations in clinical settings.

INTRODUCTION

Apoptosis plays an important role in the radiation response of both tumors and normal tissues. In several tumors such as lymphomas, apoptosis is the predominant mode of cell death after exposure to ionizing radiation (*1*). In addition, radiation-induced apoptosis is involved in the pathogenesis of radiotherapy-induced normal tissue reactions, for example in the bone marrow (*2*) or in the intestine (*3*).

Investigations of the molecular mechanisms underlying radiation-induced apoptosis have led to the conclusion that in the presence of air, reactive oxygen species (ROS) such as $OH^{\cdot}$, $O_2^{\cdot-}$ and various organic peroxyl radicals are involved in several steps of the signal transduction cascade leading to apoptosis. Radiation-induced ROS inflict the initial damage in the nuclear DNA or cellular membrane. ROS are also involved in signal transduction processes after TP53 stabilization (*4*) and are induced by the mitochondrial permeability transition (*5*).

The involvement of ROS in triggering and executing radiation-induced apoptosis offers the possibility of influencing cellular radiosensitivity by modulating antioxidant defense. Cell survival can be increased when the rate of apoptosis is reduced by treating cells with various antioxidants and thiol-containing compounds (*6, 7*). On the other hand, cell survival can be reduced when the amount of radiation-induced apoptosis is increased by reducing the cellular glutathione concentration (*8*).

Among the numerous substances that have the potential to modulate the cellular antioxidant defense and thereby influence radiosensitivity, the antioxidant vitamins E, C and beta-carotene deserve special attention for a number of reasons:

1. The antioxidant function, which results from the reaction with superoxide, hydroxyl radicals, and singlet oxygen, is well documented (*9*) along with its spectroscopic and kinetic characteristics (*10, 11*).
2. All three antioxidant vitamins are physiological compounds that have no significant toxicity when they are given at physiological concentrations.
3. Antioxidant vitamins have been administered to patients to treat side effects of radiotherapy for decades (*12–14*).

The radioprotective effect of the antioxidant vitamins has been well established. Witenberg *et al.* (*15*) found inhibition of radiation-induced apoptosis by ascorbic acid in

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HL60 cells. The radioprotective effects of beta-carotene (e.g. *16*) and vitamin E (e.g. *17*) have been demonstrated in animal models. Furthermore, there is growing evidence that reaction products of antioxidant vitamins show a pro-apoptosis effect. For instance, oxidative stress triggered by oxidized vitamin C can induce apoptosis in PC12 cells (*18*), and oxidation products of beta-carotene have been found to induce DNA strand breaks and to reduce survival in human fibroblasts (*19*). The formation of these reaction products is a consequence of the transient decay of the corresponding vitamins (*10, 11*). It therefore depends on the intracellular vitamin concentration, the presence of other antioxidant vitamins, and the process of electron transfer processes between the vitamins and their transients (*11, 20, 21*).

Since vitamins are administered to patients in a wide dose range and in various combinations, pro-apoptosis effects might significantly influence the effectiveness of these vitamins in the treatment of side effects during radiotherapy. Therefore, we studied the effects of beta-carotene, vitamin C, and alpha-tocopherol, given alone or in combination, on the induction of apoptosis in cells of the human lymphoblastic cell line MOLT-3 after exposure to ionizing radiation in air. We selected MOLT-3 cells as a model system since these cells show a high propensity to undergo apoptosis after irradiation. The effects of the vitamins were studied over a wide concentration range from 0.01 to 100 μM. To discriminate between the effects on radiation-induced primary ROS and on ROS generated during apoptosis signaling, vitamins were added either prior to or immediately after irradiation.

MATERIALS AND METHODS

Cell Culture

Cells of the human T-lymphoblastic leukemia cell line MOLT-3 (ATCC CRL 1552), initially described by Minowada *et al.* (*22*), were cultured in RPMI 1640 medium (Gibco BRL) supplemented with 15% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The cells were a gift from Dr. Shehata, Department for Haematology, University Hospital Vienna. Cultures were maintained in a humidified atmosphere containing 95%/5% CO_2 at a temperature of 37°C. The cells were kept at a density below 5×10^5/ml, and the experiments were performed at a cell density of about 3×10^5/ml.

Drug Treatment and Irradiation

Beta-carotene and alpha-tocopherol (Hoffmann-LaRoche) were solubilized in absolute ethanol. Ascorbic acid (Hoffmann-LaRoche) was dissolved in physiological saline (0.15 *M*). The vitamins were diluted with cell culture medium to give concentrations of 10 n*M*, 1 μM and 100 μM. Vitamins were added either 1 h prior to irradiation or within 5 min thereafter. In the experiments where MOLT-3 cells were irradiated without the addition of vitamins, a volume of solvent (ethanol or saline) was added to the cultures that was equal to the amount added in the experiments where vitamins were combined with radiation. Cells were irradiated at room temperature with an RT 100 X-ray machine (Philips) using 100 kV and 1.7 mm aluminum filtration. The irradiations were performed in ambient air. The dose rate was approximately 1.31 Gy/min.

Detection of Apoptosis

Eight or 24 h after irradiation, apoptotic cells were scored according to their nuclear morphology by staining with Hoechst 33342 (Sigma). Cells were treated with 10 μg/ml Hoechst 33342 in cell culture medium for 15 min at 4°C and then visualized with a fluorescence microscope (OlympusBH 2) equipped with a 320-nm excitation and a 480-nm emission filter. Cells exhibiting nuclear condensation and fragmentation were considered to be apoptotic.

Nuclear DNA fragmentation was determined by gel electrophoresis as described (*23*). Changes in the mitochondrial membrane potential were visualized using MitoSensor dye (Clontech) as described by the manufacturer. Phosphatidylserine translocation in the cell membrane was detected by Annexin V staining (*24*) using an Annexin V-FITC staining kit (Clontech) according to the instructions given by the manufacturer.

For each data point, five independent experiments were performed. About 300 Hoechst 33342-stained cells were counted in each experiment. The results, shown in Figs. 3–5, are presented as relative radioprotection, which is the mean of the background-corrected fraction of apoptotic cells found in irradiated and vitamin-treated samples divided by the background-corrected fraction of apoptotic cells found in samples that were irradiated in the absence of vitamins. The error bars represent the standard errors of the mean. Student's *t* test for paired samples was used, and points were considered to be significantly different for *P* values lower than 0.025.

Determination of Intracellular Vitamin Concentrations

MOLT-3 cells were cultured in 500-ml spinner cultures at a density around 0.5×10^6 cells/ml. Vitamins were added to the culture and the cells were harvested by centrifugation. For the determination of the vitamin C concentration, cells and medium were extracted with perchloric/metaphosphoric acid (*25*). Alpha-tocopherol and beta-carotene were extracted from the cells and from the culture medium with n-hexane (*26*). Vitamin C was determined by isocratic HPLC using a C_{18} column and a 254-nm detector exactly as described (*25*). Beta-carotene and alpha-tocopherol were determined by isocratic HPLC using a C_{18} column and a 450-nm detector for beta-carotene or a 290-nm detector for alpha-tocopherol exactly as described (*26*).

Since as no data on the intracellular distributions of the vitamins in MOLT-3 cells are available, concentrations were calculated assuming a sphere 12.5 μm in diameter (12.5 μm = mean diameter of MOLT-3 cells).

RESULTS

Irradiated MOLT-3 cells undergo apoptosis within 6 h after irradiation, exhibiting nuclear condensation and fragmentation. This can be seen in the upper panel of Fig. 1, which shows the nuclear morphology of MOLT-3 cells 8 h after irradiation with 8 Gy. Although MOLT-3 cells do not exhibit oligonucleosomal DNA fragmentation, all other hallmarks of apoptosis like mitochondrial depolarization (Fig. 1, middle panel) or phosphatidylserine externalization (Fig. 1, lower panel) can be observed.

In our experiments, untreated cultures of MOLT-3 cells exhibited a background apoptosis rate of 3.7 ± 0.33%. After irradiation the cells underwent primary apoptosis with the typical changes in nuclear morphology occurring after 6 h. After 8 h, the amount of apoptosis was proportional to dose for doses up to 6 Gy. When MOLT-3 cells were irradiated with 3 Gy, 37.0 ± 0.8% of the cells undergo apoptosis within 8 h. A dose of 3 Gy therefore leads to an

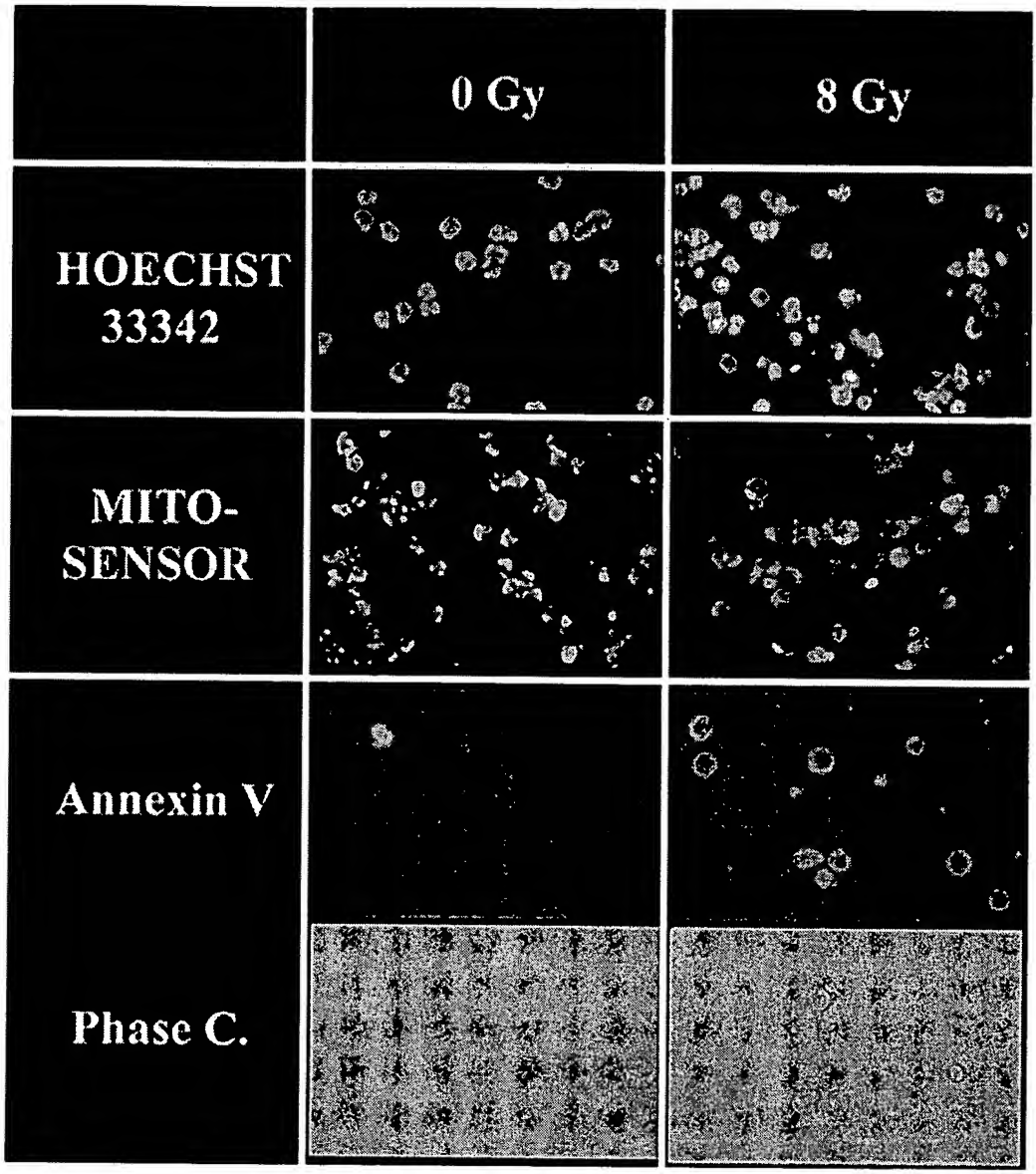

FIG. 1. Documentation of various hallmarks of apoptosis occurring in MOLT-3 cells 8 h after irradiation with a dose of 8 Gy. The upper panel shows the nuclear condensation and fragmentation visible after staining of the DNA with Hoechst 33342. The middle panel shows the loss of mitochondrial membrane potential visible by the lack of MitoSensor-stained mitochondria in the irradiated sample. The lower panel shows the Annexin V staining of apoptotic MOLT-3 cells after irradiation above a phase-contrast micrograph of the same microscope field.

amount of apoptosis that is close to the middle of the linear part of the dose–response curve, allowing optimal detection of possible protective or sensitizing effects.

Since any modulation of radiation-induced apoptosis would be expected to depend on the intracellular vitamin level, uptake of the vitamin by MOLT-3 cells was measured and is shown in Fig. 2. No detectable beta-carotene or vitamin E was found in untreated cells. As early as 15 min after addition of beta-carotene and vitamin E to the cell cultures, the intracellular vitamin concentrations were similar to the concentration in the cell culture medium. Longer incubation times did not change the intracellular vitamin concentrations significantly. In untreated cells, the intracellular vitamin C concentration was 1.3 m*M*. Fifteen minutes after the addition of 100 μ*M* vitamin C to the culture medium, the intracellular vitamin C concentration increased to 2.9 m*M*. After background correction, this is 15.9-fold above the extracellular concentration. This indicates active uptake of vitamin C, which has already been demonstrated for other leukemia cell lines (*27*). As shown in Fig. 2, the intracellular vitamin C concentration did not increase significantly after longer incubation times.

When beta-carotene, vitamin C and vitamin E were given alone or in combination at concentrations of 10 n*M*, 1 μ*M*

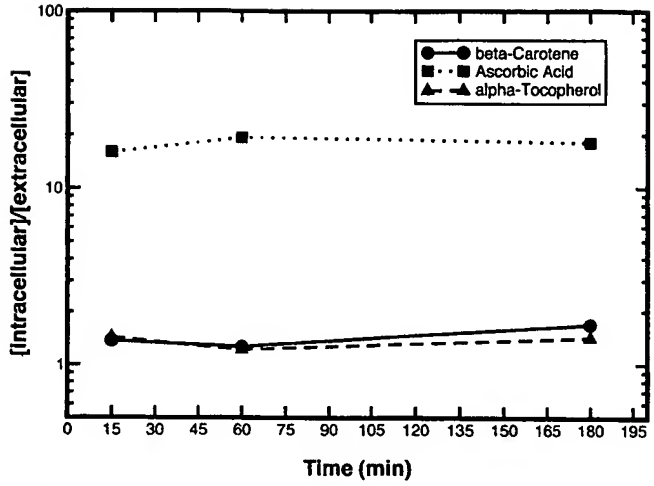

FIG. 2. Cellular uptake of the three vitamins measured at different times after the addition of the substances. The measured intracellular vitamin concentrations were corrected for the background found in untreated cells and are given as ratios of intracellular to extracellular vitamin concentrations.

or 100 μM without irradiation, there was no significant change from the background apoptosis rate. In addition, there was no significant difference in the amount of radiation-induced apoptosis in cell cultures that were irradiated with and without solvent (ethanol or saline).

The effect of a 1-h preincubation of MOLT-3 cells with beta-carotene, vitamin C and vitamin E on the amount of radiation-induced apoptosis after an absorbed dose of 3 Gy is shown in Fig. 3a. Beta-carotene and vitamin E had a significant radioprotective effect when added to the medium prior to irradiation at 10 nM and 1 μM, reducing radiation-induced apoptosis by a factor of over 1.2. In contrast, vitamin C did not influence the induction of radiation-induced apoptosis at these concentrations. At the highest concentration tested (100 μM), none of the vitamins reduced radiation-induced apoptosis. Furthermore, incubation of the cells with 100 μM vitamin C increased the amount of radiation-induced apoptosis by 22% compared to cells that received radiation only.

Although beta-carotene and vitamin E reduced the level of radiation-induced apoptosis 8 h after irradiation, it cannot be ruled out that the two vitamins simply postponed the occurrence of apoptosis. We therefore irradiated MOLT-3 cells with 1 Gy 60 min after exposure to 10 nM of the vitamins and scored the amount of apoptosis 24 h after irradiation. The data from these experiments are shown in Fig. 3a at a concentration of 8 nM. Beta-carotene and vitamin E reduced the level of radiation-induced apoptosis by roughly the same amount that was observed after 8 h. Vitamin C did not have any radioprotective effect after either 8 or 24 h.

Figure 3b shows the effect of the vitamins given immediately after irradiation. All three vitamins showed a moderate radioprotective effect at 10 nM. However, no significant alteration of the amount of radiation-induced apoptosis was observed at 1 or 100 μM. The change observed after irradiation in the presence of 100 μM vitamin C or beta-carotene was not significant.

Figure 4 shows the influence of combinations of two antioxidant vitamins on the induction of apoptosis after irradiation of MOLT-3 cells with 3 Gy. Figure 4a depicts the effect when the combinations were given 1 h prior to irradiation. At 10 nM, all combinations of two vitamins reduced the amount of radiation-induced apoptosis significantly by a factor of about 1.4. At 1 μM, all combinations

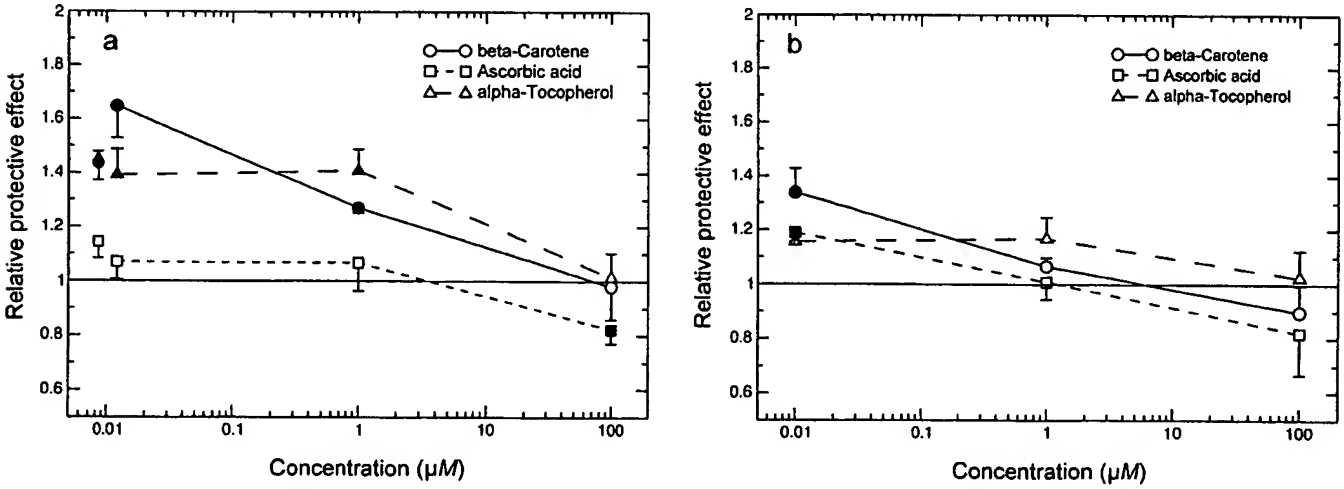

FIG. 3. Relative protective effect against radiation-induced apoptosis in MOLT-3 cells by beta-carotene, vitamin E and vitamin C. Panel a: Vitamins were given 1 h prior to irradiation. Panel b: Vitamins were added within 5 min after irradiation. The relative protective effect was calculated by dividing the rate of apoptosis found in irradiated and untreated cells by the rate of apoptosis determined in irradiated and vitamin-treated cells. The means and the standard errors of the mean of at least five independent experiments are given. Closed symbols represent data points that are significantly different ($P < 0.025$) from samples that were irradiated only. Vitamin concentrations for the data points near 10 nM were exactly 10 nM, and the points have been shifted slightly to the left (apoptosis measured after 24 h) and to the right (apoptosis measured after 8 h) for clarity.

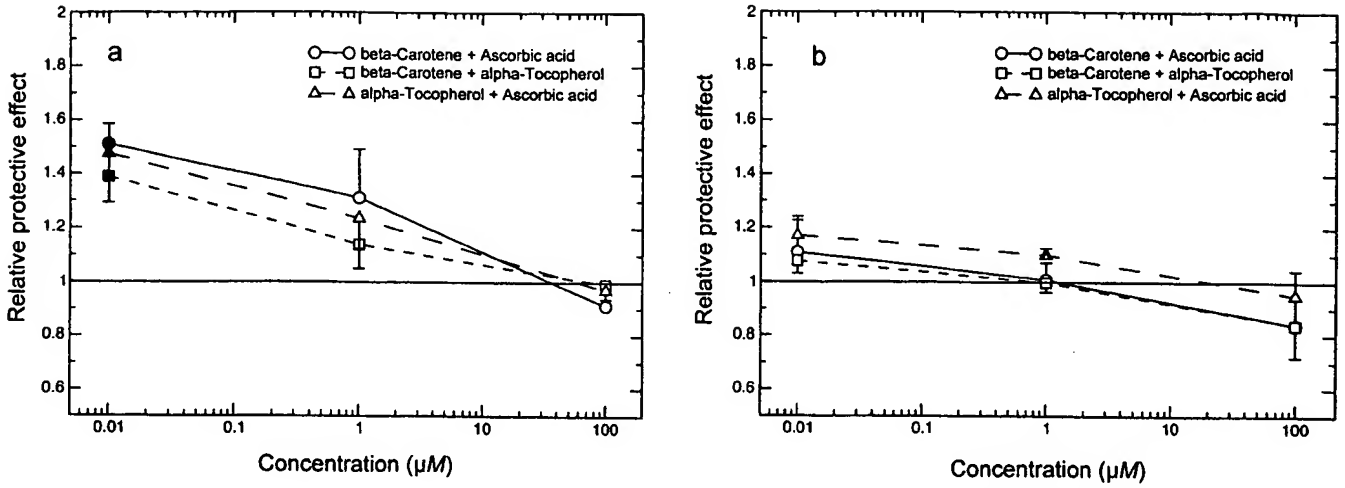

FIG. 4. Relative protective effect against radiation-induced apoptosis of all combinations of two antioxidant vitamins. Panel a: Combinations of vitamins were added prior to irradiation. Panel b: Vitamins were added immediately after irradiation. The relative protective effect was calculated as described in Fig. 2. The means and the standard errors of the mean of at least five independent experiments are given, with closed symbols representing data points showing a significant modulation of radiation-induced apoptosis.

of two vitamins showed a slight, insignificant protective effect. No modulation of radiation-induced apoptosis was observed with combinations of two vitamins at 100 μ*M*.

The effects of the adding combinations of two vitamins within 5 min after irradiation on the level of radiation-induced apoptosis are shown in Fig. 4b. The mixtures of two vitamins did not cause large changes in the amount of apoptosis compared to the level in untreated cells. However, the slight protective effect of 1.1 found with the combination of alpha-tocopherol and vitamin C at 1 μ*M* was significant.

Figure 5, shows the effects when all three of the antioxidant vitamins were added to the cell culture. Figure 5a shows the effects of the vitamins when added 1 h prior to irradiation. A significant reduction in the rate of radiation-induced apoptosis was observed at 10 n*M* and 1 μ*M*. No effect was seen at 100 μ*M* or when the combination of all three vitamins was given in all concentrations tested immediately after irradiation (Fig. 5b).

DISCUSSION

The use of antioxidant vitamins in radiotherapy to reduce radiation-induced normal tissue reactions has been reviewed (*13, 14*). Experimental data supporting use of beta-carotene, vitamin E and vitamin C for this purpose stem from numerous studies in small rodents performed mainly during the last decade. While several of these studies (e.g. *28–30*) found a significant reduction in radiation-induced chromosomal damage as measured mainly by the micro-

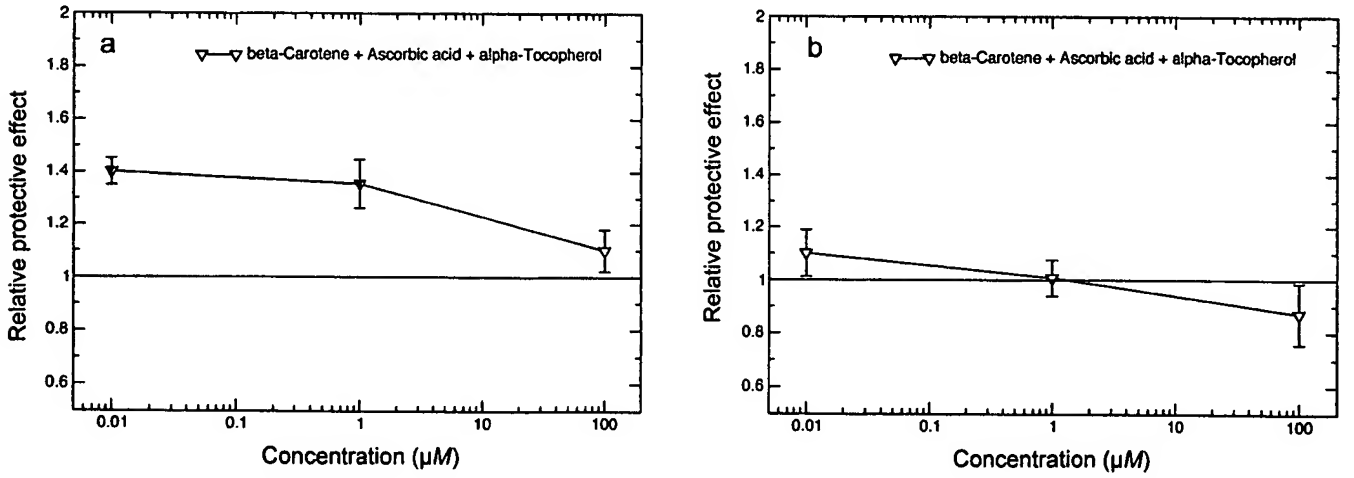

FIG. 5. Relative protective effect against radiation-induced apoptosis of the combination of all three antioxidant vitamins. Panel a: Vitamins were added 1 h prior to irradiation. Panel b: Vitamins were added immediately after irradiation. The relative protective effect was calculated as described in Fig. 2. Means and the standard errors of the mean of at least five independent experiments are given. Closed symbols represent data points showing a significant modulation of radiation-induced apoptosis ($P < 0.025$).

nucleus assay after the administration of the antioxidant vitamins, other authors present diverging results. For instance, Odagiri *et al.* (*31*) reported no reduction in radiation-induced micronucleus formation in mouse bone marrow after the administration of vitamin C, el-Nahas *et al.* (*32*) reported no effect of vitamin E in a similar experimental system, and Salvadori *et al.* (*33*) reported that the effect of vitamin E depends on the tissue. These discrepancies might have been caused by the presence of the three vitamins as physiological compounds in the experimental animals even without dietary supplementation and by the interaction with the other antioxidant defense systems present *in vivo*. Therefore, we decided to evaluate the effect of the above-mentioned vitamins in an *in vitro* system.

Reports on the interactions between ionizing radiation and antioxidant vitamins in cell culture systems have been published. Konopacka *et al.* (*34*) reported that beta-carotene, vitamin E and vitamin C significantly reduced radiation-induced micronucleus formation in stimulated human lymphocytes when given in micromolar concentrations. However, no specific effect on cell survival was reported. For the human pro-myelocytic cell line HL60, which is known to undergo apoptosis because of a radiation-induced G_2-phase block (*35*), it was reported that vitamin C reduced the rate of radiation-induced apoptosis (*15*).

In contrast, we observed no significant reduction in the rate of radiation-induced apoptosis when vitamin C was added prior to irradiation. This difference might be explained by the presence of different cell line-specific pathways leading to radiation-induced apoptosis in MOLT-3 and HL60 cells. Another explanation for the lack of a protective effect of vitamin C might be found in the observed active cellular uptake of vitamin C. The active uptake of vitamin C from the fetal bovine serum in the medium of untreated cultures as well as from the 100 μM ascorbic acid added in the treated cultures could explain the high intracellular concentrations of ascorbic acid found in control and vitamin C-treated cells. For beta-carotene and vitamin E, which do not undergo active uptake, we found no protection against radiation-induced apoptosis at the highest concentration studied. Since active uptake of vitamin C leads to a higher concentration in the cell than in the medium, the lack of a protective effect of vitamin C might be comparable to the lack of an effect seen with beta-carotene and vitamin E at higher vitamin concentrations.

We observed an increase in radiation-induced apoptosis with 100 μM vitamin C. Although there have been reports describing an induction of apoptosis by vitamin C in several cell types, including PC12 (*18*), Jurkat (*36*) and HL60 cells (*37*), we did not observe induction of apoptosis by vitamin C in unirradiated cells. Differences in the vitamin C concentration or cell line-specific factors might be responsible for the observed lack of vitamin C toxicity in unirradiated MOLT-3 cells. However, as reviewed in ref. (*38*), ascorbyl radicals might interact with radiation-induced radicals and therefore cause sensitization of cells to radiation-induced apoptosis, observed at 100 μM vitamin C.

A significant reduction in the level of radiation-induced apoptosis was observed after treatment of MOLT-3 cells with 10 nM or 1 μM beta-carotene or vitamin E prior to irradiation. Interestingly, no effect was observed when the two vitamins were added at 100 μM. No toxicity was observed in MOLT-3 cells treated with 100 μM beta-carotene or vitamin E. One might speculate that this lack of protection at high concentrations is caused by the generation of reactive compounds when the vitamins react either with radiation-induced ROS or with ROS occurring during signal transduction leading to apoptosis. Generation of reactive reaction products such as beta-carotene radical cations has been described (*10*). While the effective concentration of beta-carotene is in the range of the serum levels found in healthy individuals [0.5–1 μM (*39*)], that for vitamin E is lower than the physiological serum level [10–30 μM (*39*)], which might explain the lack of effect reported by some authors (*31, 32*).

In contrast to the effect found when vitamin C was given prior to irradiation, we observed a slight but significant reduction of radiation-induced apoptosis when 10 nM vitamin C was added immediately thereafter. A roughly similar reduction was observed for 10 nM beta-carotene and 10 nM vitamin E. This might indicate an interference of the vitamins with ROS involved in apoptotic signal transduction events, which has been observed for other antioxidants (*40*). In particular, it has been reported (*41*) that Trolox, a water-soluble vitamin E analogue, inhibited radiation-induced apoptosis in MOLT-4 cells when given at a concentration of 10 mM after irradiation. This effect was attributed to an inhibition of lipid peroxidation (*42, 41*) followed by a reduction in SAPK/JNK activation and pro-caspase 3 conversion (*43*). While this effect is consistent with our findings, the radioprotection we observed was much less pronounced than that reported for Trolox (*41, 43*) and was not observed with 100 μM vitamin E in our experiments. The most probable explanation for this difference may lie in the different subcellular distributions caused by the vastly different hydrophilicity of the two substances.

A direct effect of the vitamins when added within minutes after irradiation on the radiation-induced radicals, which consist mainly of $OH^{\cdot}$ and $O_2^{\cdot -}$, can be ruled out because the lifetimes of these species are in the microsecond range. However, the vitamins may react with longer-lived peroxyl radicals resulting from the interaction of the above-mentioned radicals with biological macromolecules. This observation is consistent with a report (*34*) describing a protective effect of the three vitamins against radiation-induced micronucleus formation when given immediately after irradiation, albeit in a higher concentration range. No protective effect was observed at vitamin concentrations of 1 and 100 μM. There was nonsignificant tendency for beta-carotene and vitamin C in this concentration range to increase the amount of radiation-induced apoptosis, perhaps

from formation of reactive or toxic vitamin reaction products.

When considering the possible radical transfer processes between the antioxidant vitamins (reviewed in ref. *44*) and the unexpected effects observed when vitamins were used in combination for chemoprevention of cancer in clinical settings (*45*), it is interesting to note that the effects we observed for vitamin combinations *in vitro* were very similar to those of the individual substances. In particular, the reduction of radiation-induced apoptosis was in the range of about 1.4 no matter which combination was given prior to irradiation at a concentration of 10 n*M* for each vitamin. This reduction is in the same range as that observed for beta-carotene or vitamin E alone, arguing against any additive effect. The lack of an additive effect is remarkable since vitamin C can be found in the cytoplasm and nucleus while beta-carotene and vitamin E are localized in the membrane compartment of the cells and therefore are considered to detoxify different kinds of ROS.

There has been some controversy about the potential risks or benefits of administering antioxidant vitamins during radiotherapy. As summarized recently (*46*), administration of antioxidant vitamins could have several very different consequences:

1. It might improve the outcome of radiotherapy through a direct growth inhibitory effect on tumor cells and might allow a higher dose to be delivered to the target volume by exerting a radioprotective effect on normal tissues. In addition, there might also be a radiosensitizing effect on tumor cells.
2. It might reduce the efficiency of radiotherapy by scavenging radiation-induced ROS not only in normal tissues but also in tumor cells and therefore increase the survival of malignant cells. In addition, this radioprotective effect might be more pronounced in tumor cells.

Although it was not the aim of our study to decide between these viewpoints, we found that the protection by beta-carotene and vitamin E against radiation-induced apoptosis was highly dependent on concentration and occurred only in the micromolar and sub-micromolar concentration range. This suggests that low extracellular vitamin concentrations could lead to the tumor cell radioprotection mentioned in the second hypothesis, while a higher extracellular vitamin concentration would not cause such a protective effect and could even increase radiation-induced apoptosis, as assumed by the first hypothesis. Therefore, a careful consideration of vitamin concentrations is required when vitamins are given to patients receiving radiotherapy.

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March 24, 2006

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MAIL STOP APPEAL BRIEF-PATENTS
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Re: U. S. Patent Application Serial No. 10/806,494 entitled, "Methods for Preventing Photodamaged Skin by Administering Selegiline or Desmethylselegiline" by Mark G. Resnick
(Our Ref: SOM700/4-9(A)8CON2US/64001)

Dear Sir:

In response to the Notification of Non-Compliant Appeal Brief, Appellant submits the following:

1. Corrected Appeal Brief, including Evidence Appendix Exhibits 1-3;

2. Credit Card Authorization form; and

3. Postcard.

The Commissioner is requested to consider this statement as a petition for an extension of time of one month, bringing the due date for reply to March 24, 2006. The appropriate fee of $120 according to 37 CFR 1.17(a)(1) for a one month extension of time is enclosed. No further fees are believed to be due with the filing of this Response to the Notification of Non-Compliant Appeal Brief and submission of the corrected Appeal Brief. If the fee is missing or should any other fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason, the Commissioner is authorized to deduct said fees from VINSON & ELKINS L.L.P. Deposit Account No. 22-0365/SOM700/4-009(A)8CON2/64001.

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Very truly yours,

Margaret J. Sampson

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